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2. Patent application number *(The Patent Office will fill in this part)* 0222846.8

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P01/7700 0.00-0222846.8

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

Yen Choo
10 Sydney Street
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Patents ADP number *(if you know it)* 08475899001

If the applicant is a corporate body, give the country/state of its incorporation

4. Title of the invention — Cell Culture

5. Name of your agent *(if you have one)* — D Young & Co

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

21 New Fetter Lane
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Patents ADP number *(if you know it)* 59006

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Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

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11. I/We request the grant of a patent on the basis of this application.

Signature D Young & Co Date 03 October 2002

D Young & Co (Agents for the Applicants)

12. Name and daytime telephone number of person to contact in the United Kingdom — Antonio Maschio — 023 8071 9500

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Cell Culture

Field of the Invention

The invention relates to cell culture, and in particular to the culture of pluripotent cells, totipotent cells and stem cells and the regulation of their development through modulation of cell culture conditions. The invention relates to the use of multiple culture steps under a plurality of conditions to modulate differentiation pathways in cells and provides methods for determining the effect of diverse multiple culture step regimes on cell growth and differentiation.

Background to the Invention

Over recent years cell culture has become a core technology in the life sciences. Cell culture provides the basis for studying the proliferation and differentiation of cells, and the formation of biological products. It has also provided the means to study the regulation of these processes, from the genetic level - whether in isolation or in whole transgenic animals – down to the level of individual protein molecules. Notwithstanding its enormous contribution to the current state of biology, in many respects cell culture remains a developing discipline, albeit an unusually exciting science ultimately offering the possibility of genetic therapy and tissue engineering.

An important goal of cell culture is to be able to grow a wide variety of cells in vitro. The list of different cell types that can be grown in culture is extensive (see www.atcc.org), includes representatives of most cell types, and is continually increasing as more and more culture conditions are discovered. Despite the steady progress in the field, the method of determining suitable culture conditions for new cell types remains totally empirical: growth conditions are almost always discovered by trial and error. The choice of starting point will often be based on what was previously used by others for similar cells, or even what is currently being used in the laboratory for different cells. Many times these will simply be completely inadequate, and a process of trial and error must begin anew. Even when new culture conditions are successful, it is worthwhile remembering that adaptations of previous protocols will have introduced a historical bias to the experiment. For instance, much of the early tissue culture experiments made extensive use of fibroblasts, and to this date most standard cell culture conditions favour growth of cells derived from the mesoderm (fibroblasts, endothelium, myoblasts). The development

of selective growth media for epithelial and other cell types based on these conditions was a challenge. For some of these cell types it is now known that serum – a normal component of many culture media for mesodermal cells – actually inhibits growth. One aspect of the invention described herein is a method for developing suitable culture conditions which allow for the growth of particular cell types.

Apart from conditions that favour cell proliferation, a particularly important step in modern tissue culture is to be able to control or direct the differentiation of cells towards a particular phenotype. As propagation of cell lines requires that the cell number increases, the vast majority of culture conditions have been developed to favour maximal cell proliferation. It is not surprising that these conditions are not conducive to cell differentiation, where cell growth is often limited or even abolished. The conditions which favour cell proliferation are generally low cell density, low Ca^{2+} concentration, and the presence of growth factors such as epidermal growth factor (EGF), fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF). On the other hand, cytostasis and differentiation are promoted in conditions of high cell density, high Ca^{2+} concentration and the presence of differentiation inducers such as hormones (e.g. hydrocortisone), paracrine factors (e.g. IL-6, KGF, NGF), retinoids and even planar polar compounds such as dimethylsulphoxide (DMSO). Hence different conditions may be required for propagation and differentiation of a particular cell line, and of course these respective conditions may differ between cells of different lineages. A second aspect of the invention described herein is a method for discovering suitable culture conditions which allow for the selective differentiation of cells.

There is a need in the art for improved techniques for culturing cells, and methods for discovering and implementing such techniques for cellular growth and differentiation.

Summary of the Invention

The present invention provides novel cell culture techniques which are based on the perception that cell culture is better approached as a dynamic process involving serial culture steps performed in a defined sequence to achieve a desired effect. The invention recognises that sequential exposure to selected agents may be exploited to modulate the differentiation of cells and thus achieve a level of control over cellular differentiation not previously attainable by conventional techniques.

The invention addresses the problem that cell culture techniques involving a plurality of steps and agents are difficult if not impossible to determine by conventional experimentation, which in the prior art has involved trial and error. Empirical determination of tissue culture conditions in complex, multi-stage procedures is not feasible in practice.

In a first aspect, the invention provides a method for determining the effect of a plurality of culture conditions on a cell, comprising the steps of:

(a) providing a first set of groups of cell units each comprising one or more cells, and exposing said groups to desired culture conditions;

(b) pooling two or more of said groups to form at least one second pool;

(c) subdividing the second pool to create a further set of groups of cell units;

(d) exposing said further groups to desired culture conditions;

(e) optionally, repeating steps (b) – (d) iteratively as required; and

(f) assessing the effect on a given cell unit of the culture conditions to which it has been exposed.

The invention addresses both the proliferation and differentiation of cells. Preferably, the invention is used to identify conditions which result in cellular differentiation. For example cells may be induced to differentiate along a desired developmental pathway, by subjecting the cells to appropriate culture conditions. The timing of the changing of culture conditions is also exploited to better define the developmental fate of the cells.

Culture conditions include growth media, agents present in growth media, temperature regimes, substrates, atmospheric conditions, physical cell handling and the like.

The above method of the invention, known as split-pool culturing, allows cells to be subjected to a series of culture conditions, and exposed to a series of agents in culture media, in a systematic and highly productive manner.

Although repetitive cycles of splitting and pooling may be used highly efficiently, in a similar manner to combinatorial chemistry protocols, given the necessary processing power protocols involving sequential splitting without repooling may be used. The disadvantage of such protocols is that they quickly generate a very large number of separate samples, which have been handled differently. The advantage, however, is that each sample does not require laborious deconvolution, since the cell units therein

have only been exposed to one set of conditions. Accordingly, given suitable sample handling facilities, a splitting approach can yield rapid results.

The invention accordingly provides, in a further aspect, a method for determining the effect of a plurality of culture conditions on a cell, comprising the steps of:

(a) providing a first set of groups of cell units each comprising one or more cells, and exposing said groups to desired culture conditions;

(b) subdividing one or more of said groups to create a further set of groups of cell units;

(c) exposing said further groups to further desired culture conditions;

(d) optionally, repeating steps (b) – (c) iteratively as required; and

(e) assessing the effect on a given cell unit of the culture conditions to which it has been exposed.

The invention employs cell units. Such units may be single cells, but are advantageously colonies of two or more cells, which are arranged in such a form that they are resistant to disruption even during split pool culturing procedures. For instance, the cells may be cultured on a solid substrate, such as beads, as described in more detail below.

Advantageously, the cell units are labelled. Labelling allows the following of the culture conditions to which the cells have been exposed; thus, any given cell unit can have its label read in order to determine how it has been derived from the starter cell pool or culture. Labels may take any of a variety of forms, including nucleic acid labels, radiofrequency encoded tags and spatial encoding of cell units on a surface or matrix.

The method of the invention allows thousands or millions of cell culture conditions and reagents to be tested, in a multiplexed high-throughput assay, to determine the conditions necessary to achieve the differentiation and/or proliferation of cells as desired.

In a further aspect, the invention provides a method for identifying a gene which influences cell proliferation and/or differentiation, comprising the steps of:

a) determining the effect of one or more culture conditions on a cell unit, in accordance with the foregoing aspect of the invention;

b) analysing gene expression in said cell units when exposed to said culture conditions; and

c) identifying genes which are differentially expressed under desired culture conditions.

Advantageously, the culture conditions used cause a change in the differentiation state and/or rate of proliferation of the cells; these culture conditions are selected for the production of cells in which gene expression is analysed. Gene expression may conveniently be analysed using array technology, which is widely available from suppliers such as Affymetrix.

In another aspect, the invention provides a method for producing a nucleic acid which encodes a gene product which influences cell proliferation and/or differentiation, comprising identifying a gene as above, and producing at least the coding region of said gene by nucleic acid synthesis or biological replication.

In a further aspect, there is provided a method for inducing differentiation and/or proliferation in a cell, comprising the steps of:

a) identifying one or more genes which are differentially expressed in association with cell differentiation and/or proliferation in accordance with the invention; and

b) modulating the expression of said one or more genes in the cell.

The expression of the genes in the cell can be modulated by, for example, transfecting or otherwise transferring the gene into the cell such that it is overexpressed in a transient or permanent manner. Alternatively, the expression of the endogenous gene may be altered, such as by targeted enhancer insertion or the administration of exogenous agents which cause an increase in expression of the gene. Moreover, the product of the gene may itself be administered to the cell to achieve the same result.

In a still further aspect, the invention provides a method for identifying the differentiation state of a cell, comprising the steps of:

a) identifying one or more genes which are differentially expressed in association with cell differentiation as set forth above; and

b) detecting the modulation of expression of said one or more genes in a cell, thereby determining the differentiation state of said cell.

Advantageously, the genes employed in this analysis encode extracellular markers, which may be detected for instance by immunoassay.

The invention further provides a method for producing a differentiated cell, comprising the steps of:

a) determining the effect of one or more culture conditions on a cell unit, in accordance with the foregoing aspect of the inventnion;

b) exposing a pluripotent or totipotent cell to culture conditions which induce the desired differentiation; and

c) isolating the desired differentiated cell.

Differentiated cells, particularly partly differentiated, pluripotent cells, are useful in cellular therapies and other procedures in which cells of a defined lineage are required.

There is also provided a method for identifying an agent which is capable of inducing cell differentiation, comprising the steps of:

a) determining the effect of one or more agents on a cell unit, in accordance with the foregoing aspect of the invention; and

b) identifying those agent(s) which induce the desired differentiation in the cell units.

Agents identified in accordance with the invention may be synthesised by conventional or other techniques, and used in methods for differentiating or proliferating cells for example as described herein.

The invention moreover broadly provides methods of culturing stem cells, and differentiated cells derived from stem cells in vitro, adherent to microcarriers, such as beads. Microcarrier culture has significant advantages, including the scale-up of cultures, and also allows units of stem cells to be exposed to selected culture conditions as required in order to obtain the desired growth and/or differentiation conditions. In the broadest embodiment, therefore, the invention provides a method for culturing stem cells and differentiated cells derived from stem cells in vitro, comprising growing said cells adhered to a microcarrier or bead.

Advantageously, the culture is subjected to at least one change of culture conditions. Preferably, they are subjected to 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more different culture conditions. Preferably, said change of culture conditions comprises a change of medium.

The invention moreover provides methods for culturing stem cells in which pools of stem cells are created and subdivided as described for the preceding embodiments of the invention. Thus, in one aspect, there is provided a method for culturing stem cells and differentiated cells derived from stem cells in vitro, comprising the steps of:

a) combining one or more cultures of cells grown under different conditions; and

b) culturing the cells under common conditions.

In further embodiment, there is provided a method for culturing stem cells and differentiated cells derived from stem cells in vitro, comprising the steps of:

a) incubating a stem cell culture; and

b) splitting said culture into two or more groups of stem cells, and culturing said group of stem cells under two or more different sets of culture conditions.

Preferably, the cells are exposed to 3, 4, 5, 6, 7, 8, 9, 10 or more different culture conditions. The culture conditions used advantageously comprise a change of medium.

Advantageously, cells are cultured in cell units, each cell unit comprising one or more cells. For example, the cell units are single cells.

However, each cell unit can comprise one or more cells adherent to or bounded by a solid substrate, such as a microcarrier or bead. Further solid substrates include a well or medium-permeable barrier.

Methods for culturing stem cells according to the invention may be scaled up in suitable bioreactors. For example, the method of the invention may be practised using more than 50g dry weight of microcarrier.

Brief Description of the Figures

Figure 1 shows an example of split-pool cell culture performed over two rounds. A group of cell units is obtained by growing under condition A. Cell units are depicted as spheres in which are written the culture conditions to which they have been exposed. The cell units are split randomly into three aliquots which are cultured for two days under different growth conditions denoted B, C or D. The cell units are subsequently pooled and split once more into three aliquots which are again grown under conditions B, C or D. After

two rounds of this protocol, it can be seen that the various cell groups have been exposed to all possible combinations of cell culture conditions.

Detailed Description of the Invention

Definitions

Culture Conditions As used herein, the term "culture conditions" refers to the environment which cells are placed in or are exposed to in order to promote growth or differentiation of said cells. Thus, the term refers to the medium, temperature, atmospheric conditions, substrate, stirring conditions and the like which may affect the growth and/ or differentiation of cells. More particularly, the term refers to specific agents which may be incorporated into culture media and which may influence the growth and/or differentiation of cells.

Cell A cell, as referred to herein, is defined as the smallest structural unit of an organism that is capable of independent functioning, or a single-celled organism, consisting of one or more nuclei, cytoplasm, and various organelles, all surrounded by a semipermeable cell membrane or cell wall. The cell may be prokaryotic, eukaryotic or archaebacterial. For example, the cell may be a eukaryotic cell. Mammalian cells are preferred, especially human cells. Cells may be natural or modified, such as by genetic manipulation or passaging in culture, to achieve desired properties. In the present invention, cells are typically pluripotent or totipotent. A stem cell is defined in more detail below, and is a pluripotent cell capable of transdifferentiation into more than one differentiated cell type. Stem cells may be differentiated *in vitro* to give rise to differentiated cells, which may themselves be pluripotent, or may be terminally differentiated. Cells differentiated *in vitro* are cells which have been created artificially by exposing stem cells to one or more agents which promote cell differentiation.

Cell unit A group of cells, which may be a group of one. Pools of cell units may be sorted, subdivided and handled without dissociating the cell units themselves, such that the cell unit behaves as a colony of cells and each cell in the cell unit is exposed to the same culture conditions. For example, a cell unit may be a bead to which is adhered a group of cells.

Totipotent A totipotent cell is a cell which may differentiate into another cell of any cell lineage. Thus, any desired cell may be derived, by some means, from a totipotent cell.

Pluripotent A pluripotent cell is a cell which may differentiate into more than one, but not all, cell types.

Label A label, as used herein, is a means to identify a cell unit and thereby determine a culture condition, or a sequence of culture conditions, to which it has been exposed. Thus, a label may be a group of labels, each added at a specific culturing step; or a label which is modified according to the culturing steps to which the cell unit is exposed; or simply a positional reference, which allows the culturing steps used to be deduced. A label may also be a device that reports or records the location of a cell unit at any one time.

Exposure to culture conditions A cell is exposed to culture conditions when it is placed in a medium, or under conditions in which, the culture conditions are able to affect the growth or differentiation of the cell. Thus, if the culture conditions comprise culturing the cell in a medium, the cell is placed in the medium for a sufficient period of time for it to have an effect. Likewise, if the conditions are temperature conditions, the cells are cultured at the desired temperature.

Pooling The pooling of one or more groups of cell units involves the admixture of the groups to create a single group or pool which comprises cell units of more than one background, that is that have been exposed to more than one different sets of culture conditions. A pool may be subdivided further into groups, either randomly or non-randomly; such groups are not themselves "pools" for the present purposes, but may themselves be pooled by combination, for example after exposure to different sets of culture conditions.

Proliferation Cell growth and cell proliferation are used interchangeably herein to denote multiplication of cell numbers without differentiation into different cell types or lineages. In other words, the terms denote multiplication of cell numbers.

Differentiation Cell differentiation is the development, from a cell type, of a different cell type. For example, a pluripotent or totipotent cell may differentiate into a neural cell. Differentiation may be accompanied by proliferation, or may be independent thereof.

Differentiation state The differentiation state of a cell is the level to which a cell has differentiated along a particular pathway or lineage.

Gene A gene is a nucleic acid which encodes a gene product, be it a polypeptide or an RNA gene product. As used herein, a gene includes at least the coding sequence which encodes the gene product; it may, optionally, include one or more regulatory regions necessary for the transcription and/or translation of the coding sequence.

Gene Product A gene product is typically a protein encoded by a gene in the conventional manner. However, the term also encompasses non-polypeptide gene products, such as ribonucleic acids, which are encoded by the gene.

Nucleic acid synthesis Nucleic acids may be synthesised according to any available technique. Preferably, nucleic acid synthesis is automated. Moreover, nucleic acids may be produced by biological replication, such as by cloning and replication in bacterial or eukaryotic cells, according to procedures known in the art.

Differential Expression Genes which are expressed at different levels in response to cell culture conditions can be identified by gene expression analysis, such as on a gene array, by methods known in the art. Genes which are differentially expressed display a greater or lesser quantity of mRNA or gene product in the cell under the test conditions than under alternative conditions, relative to overall gene expression levels.

Transfection Genes may be transfected into cells by any appropriate means. The term is used herein to signify conventional transfection, for example using calcium phosphate, but also to include other techniques for transferring nucleic acids into a cell, including transformation, transduction, electroporation and the like.

Modulation The term modulation is used to signify an increase and/or decrease in the parameter being modulated. Thus, modulation of gene expression includes both increasing gene expression and decreasing gene expression.

Stem Cells

Stem cells are described in detail in *Stem Cells: Scientific Progress and Future Research Directions*. Department of Health and Human Services. June 2001. http://www.nih.gov/news/stemcell/scireport.htm. The contents of the report are herein incorporated by reference.

Stem cells are cells that are capable of differentiating to form at least one and sometimes many specialised cell types. The repertoire of the different cells that can be formed from stem cells is thought to be exhaustive; that is to say it includes all the different cell types that make up the organism. Stem cells are present throughout the lifetime of an organism, from the early embryo where they are relatively abundant, to the adult where they are relatively rare. Stem cells present in many tissues of adult animals are important in normal tissue repair and homeostasis.

The existence of these cells has raised the possibility that they could provide a means of generating specialised functional cells that can replace dead or non-functioning cells in diseased tissues. As undifferentiated stem cells transplanted into animals frequently give rise to tumours, it is envisaged that differentiating the stem cells into more specialised cells will be a pre-requisite for cell replacement therapy. The list of diseases for which this may provide therapies includes Parkinson's disease, diabetes, spinal cord injury, stroke; chronic heart disease, end-stage kidney disease, liver failure and cancer. In order for cell replacement therapy to become feasible at least two major breakthroughs in stem cell research are required. First, conditions for growing stem cells to sufficient numbers need to be developed, so that therapeutically relevant doses of cells can be manufactured. Ideally large-scale cultures would be possible, providing material to treat multiple patients. Secondly, it will be necessary to discover conditions for differentiating stem cells in to the particular, specialised cell types required for different diseases.

It is clear that the key to these obstacles lies in devising suitable methods of cell culture for stem cells and their derivatives. However, because of the reasons explained above – namely the laborious process of trial and error involved in the evolution of cell culture techniques - the task is particularly difficult. Hence one of the applications of the invention described herein is in the elucidation of techniques for the growth and differentiation of stem cells.

Types of stem cell

There is still considerable debate about what constitutes a stem cell, however for the purposes of this discussion a key characteristic is the ability to differentiate into a different cell type. Examples of stem cells are given below.

Different stem cells have differing potential to form various cell types: spermatogonial stem cells are unipotent as they naturally produce only spermatozoa, whereas haematopoietic stem cells are multipotent, and embryonic stem cells are thought to be able to give rise to all cell types and are said to be totipotent or pluripotent.

To date three types of mammalian pluripotent stem cell have been isolated. These cells can give rise to cell types that are normally derived from all three germ layers of the embryo (endoderm, mesoderm and ectoderm). The three types of stem cell are: embryonal carcinoma (EC) cells, derived from testicular tumours; embryonic stem (ES) cells, derived from the pre-implantation embryo (normally the blastocyst); and embryonic germ (EG) cells derived from the post-implantation embryo (normally cells of the foetus destined to become part of the gonads). These cells are receiving particular attention in the effort to direct differentiation, precisely because they are pluripotent.

Stem cells are also present in the adult organism. An adult stem cell is an undifferentiated cell that occurs in a differentiated (specialised) tissue, renews itself, and can differentiate to yield more specialised cells. Recently it has been shown that adult stem cells are capable of plasticity, that is to say they can differentiate to yield cell types that are not characteristic of the tissue in which they reside, nor indeed of the germ layer from which that tissue originates For example, it has been shown that blood stem cells (derived from mesoderm) can differentiate into neurons (normally derived from ectoderm). Toma *et al.*(2001, *Nature Cell Biol.* 3, p778–784) have recently described the identification and isolation of a new type of stem cell that was derived from the dermis of the skin. These stem cells were termed skin-derived precursor (SKP) cells. The SKP cells could be induced to differentiate by culturing on poly-lysine and varying the concentration of serum in the culture medium. In the absence of serum they differentiate into neurons and glial cells; with addition of 3% serum they differentiate into smooth-muscle cells; and increasing the serum to 10% causes the SKP cells to differentiate into adipocytes. Adult stem cells have so far been reported in tissues as diverse as the

nervous system, the bone marrow and blood, the liver, skeletal muscle, the skin and digestive system.

In addition to the adult stem cells there are numerous types of progenitor or precursor cells. These are partially specialised cells that occur in probably all of the tissues of the body – they are capable of differentiating but differ from stem cells in that their repertoire is not as broad, and by definition they are not capable of self-renewal. Recent evidence even suggests that differentiated cell types are capable of changing phenotype. This phenomenon, termed transdifferentiation, is the conversion of one differentiated cell type to another, with or without an intervening cell division. It used to be generally accepted that the terminal differentiated state is fixed, but it is now clear that differentiation can sometimes be reversed or altered. The appearance of hepatocytes in the pancreas is one well-documented example of transdifferentiation. Hepatocytes appear in the pancreas of hamsters or rats in response to various experimental treatments — for example, treatment of rats with a copper-deficient diet, or after transplantation of epithelial cells, and in transgenic mice that overexpress keratinocyte growth factor in the pancreatic islets. The reverse transdifferentiation (liver to pancreatic exocrine cell) is induced in the livers of rats after treatment with polychlorinated biphenyls. In vitro protocols are now available in which cell lines can be induced to transdifferentiate (see Shen, Slack & Tosh, 2000, *Nature Cell Biol.* vol 2, p. 879–887). Finally, there have been reports of specialised cell types that can de-differentiate to yield stem-like cells with the potential to differentiate into further cell types.

Stem cell growth and differentiation

An important property of stem cells is their ability to divide symmetrically in culture, giving rise to two daughter cells that are exact copies of the stem cell from which they were derived. This allows stem cells to be expanded in culture in their undifferentiated state, producing enough material for biological studies or even cell therapy. The means by which stem cells are able to do this is naturally the subject of intensive research, yet few of the factors that promote stem cell renewal are known. Typically, pluripotent stem cell lines are maintained on mitotically inactive feeder layers of fibroblasts, or medium conditioned by such cells. It is assumed that feeder cells either remove/neutralise some unknown factor from the culture medium, or otherwise they provide a factor that suppresses the differentiation and promotes the self-renewal of stem cells. One such factor is leukaemia inhibitory factor (LIF), a member of the cytokine family related to IL-6,

which is capable of promoting mouse ES cell self-renewal in the absence of feeder cells. Stem cells grown in the absence of feeder cells (and/or LIF) often differentiate spontaneously and haphazardly, producing a mixture of differentiated cell types.

The factors that influence stem cell self-renewal may either be stimulatory or inhibitory and may function extracellularly or intracellularly. In the case of the secreted factor LIF, it is known that its extracellular receptor is gp 130, and that activation of this protein is both necessary and sufficient for inhibiting murine ES cell differentiation. Within the cell, a crucial downstream effector of gp130 is the signal transducer and activator of transcription-3 (STAT-3). Another molecule which is particularly important in maintaining stem cell pluripotency is the transcription factor Oct-4, which when downregulated artificially leads to the loss of the pluripotent state in ES cells or mice. Other signalling molecules that naturally inhibit ES cell self-renewal, such as the mitogen-activated protein kinase, have also been elucidated. A major goal of stem cell research will be the discovery of natural and synthetic factors, drugs, polypeptides, genes, oligonucleotides, tissue culture media and conditions, specific conditioned media, feeder cells, and other stimuli that have the effect of promoting the expansion and retaining the differentiation potential of various types of stem cell. This includes adult stem cells, which at present have not been expanded appreciably in cell culture.

The second great challenge of stem cell research is to direct the differentiation of stem cells to particular cell types which are functional, can replace cells lost in various disease states, and result in a positive clinical outcome. Coaxing stem cells to begin differentiating is actually a fairly straightforward process. For instance, ES cells removed from feeder cultures and grown to confluence on an adherent substrate will begin to differentiate spontaneously. Similarly, ES cells removed from feeder cultures and grown on a non-adherent substrate will form embryoid bodies - clusters of undifferentiated and partially differentiated cells from all three germ layers. These cells can be subsequently dissociated and plated in monolayer culture, and exposed to factors that promote directed differentiation. Cultures exposed to these factors are more likely to be populated by one or two types of differentiated cell, compared to embryoid bodies or untreated cultures of differentiating cells which comprise mixtures of many different cell types. Nevertheless, few if any conditions have been devised thus far that produce substantially pure cultures of differentiated cells. In addition it is not clear if any of the protocols devised for stem cell differentiation yield cells that are suitable for cell replacement

therapy – it may be that the cells have not terminally differentiated into the precise phenotype required, or that the differentiated cells are no longer viable in vivo.

The factors that have been used to induce directed differentiation of stem cells include: retinoic acid, epidermal growth factor (EGF), bone morphogenic proteins (BMPs), basic fibroblast growth factor (bFGF), activin-A, transforming growth factor beta-1 (TFG β-1), hepatocyte growth factor, nerve growth factor, sonic hedgehog (SHH), interleukin-3 (IL-3), interleukin-6 (IL-6), granulocyte macrophage colony stimulating factor (GM-CSF), erythropoietin, vitamin D3, dexamethasone, β-mercaptoethanol, butylated hydroxyanisole, 5-azacytidine, DMSO, insulin, thyroid hormone (T3), LIF, foetal calf serum, vascular endothelial growth factor (VEGF), steel factor, low oxygen concentration, ascorbic acid, β-glycerophosphate, nicotinamide, platelet derived growth factor (PDGF), cAMP, various cell adhesion molecules and substrates, and others. In addition to these defined factors, it is likely that undefined extracts, such as conditioned media, human and animal tissue homogenates, or plant extracts can be used to direct stem cell differentiation. Progressive fractionation of these undefined extracts may yield active fractions or even pure components with high potency. These factors can be added to the growth medium used in a particular experiment, either alone, or in combination, or in a defined order which is crucial to the experimental result.

Many systems that have been devised for the differentiation of stem cells in vitro are complex multi-stage procedures, in which the precise nature of the various steps, as well as the chronology of the various steps, are important. For instance, Lee et al (2000, Nature Biotechnology, vol. 18, p 675-679) used a five stage protocol to derive dopaminergic neurons from mouse ES cells. Undifferentiated ES cells were expanded on gelatin-coated tissue culture surface in ES cell medium in the presence of LIF (stage 1). Embryoid bodies were generated in suspension cultures for 4 days in ES cell medium (stage 2). Nestin-positive cells were selected from embryoid bodies in ITSFn medium for 8 days after plating on tissue culture surface (Stage 3). Nestin-positive cells were expanded for 6 days in N2 medium containing bFGF/laminin (Stage 4). Finally the expanded neuronal precursor cells were induced to differentiate by withdrawing bFGF from N2 medium containing laminin (Stage 5).

However it is not only the sequence and duration of the various steps or the series of addition of different factors that is important in the determination of cell differentiation. As embryonic development occurs through gradients of signalling factors that impart

positional information, it is to be expected that the concentration of a single signalling factor, and also the relative concentration of two factors, will be important in specifying the fate of a cell population in vitro and in vivo. Factor concentrations vary during development and stem cells respond differently to different concentrations of the same molecule. For instance, stem cells isolated from the CNS of late stage embryos respond differently to different concentrations of EGF: low concentrations of EGF result in a signal to proliferate, while higher concentrations of EGF result in proliferation and differentiation to astrocytes.

Many of the factors that influence self-renewal and differentiation of stem cells are naturally-occurring molecules. This is to be expected, as differentiation is induced and controlled by signalling molecules and receptors that act along signal transduction pathways. However, by the same token, it is likely that many synthetic compounds will have an effect on stem cell differentiation. Such synthetic compounds that have high probability of interacting with cellular targets within signalling and signal transduction pathways (so called drugable targets) are routinely synthesised, for instance for drug screening by pharmaceutical companies. Once known, these compounds can be used to direct the differentiation of stem cells ex vivo, or can be administered in vivo in which case they would act on resident stem cells in the target organ of a patient.

Common variables in tissue culture

In developing conditions for the successful culture of a particular cell type it is often important to consider a variety of factors.

One important factor is the decision of whether to propagate the cells in suspension or as a monolayer attached to a substrate. Most cells prefer to adhere to a substrate although some, including transformed cells, haematopoietic cells, and cells from ascites, can be propagated in suspension.

Assuming the culture is of adherent cells, an important factor is the choice of adhesion substrate. Most laboratories use disposable plastics as substrates for tissue culture. The plastics that have been used include polystyrene (the most common type), polyethylene, polycarbonate, Perspex, PVC, Teflon, cellophane and cellulose acetate. It is likely than any plastic can be used, but many of these will need to be treated to make them wettable and suitable for cell attachment. Furthermore it is very likely that any suitably prepared

solid substrate can be used to provide a support for cells, and the substrates that have been used to date include glass (e.g. alum-borosilicate and soda-lime glasses), rubber, synthetic fibres, polymerised dextrans, metal (e.g. stainless steel and titanium) and others.

Some cell types, such as bronchial epithelium, vascular endothelium, skeletal muscle and neurons require the growth substrate to be coated with biological products, usually extracellular matrix materials such as fibronectin, collagen, laminin, polylysine or others. The growth substrate and the method of application (wet or dry coating, or gelling) can have an effect on the growth and differentiation characteristics of cells, and these must be determined empirically as discussed above.

Probably the most obviously important of the variables in cell culture is the choice of culture medium and supplements such as serum. These provide an aqueous compartment for cell growth, complete with nutrients and various factors, some of which have been listed above, others of which are poorly defined. Some of these factors are essential for adhesion, others for conveying information (e.g. hormones, mitogens, cytokines) and others as detoxificants. Commonly used media include RPMI 1640, MEM/Hank's salts, MEM/Earle's salts, F12, DMEM/F12, L15, MCDB 153, and others. The various media can differ widely in their constituents - some of the common differences include sodium bicarbonate concentration, concentration of divalent ions such as Ca and Mg, buffer composition, antibiotics, trace elements, nucleosides, polypeptides, synthetic compounds, drugs, etc. It is well known that different media are selective, meaning they promote the growth of only some cell types. Media supplements such as serum, pituitary brain and other extracts, are often essential for the growth of cells in culture, and in addition are frequently responsible for determining the phenotype of cells in culture, i.e. they are capable of determining cell survival or directing differentiation. The role of supplements in cell differentiation is complex and depends on their concentration, the time point at which they are added to the culture, the cell type and medium used. The undefined nature of these supplements, and their potential to affect the cell phenotype, have motivated the development of serum-free media. As with all media, their development has come about largely by trial and error, as has been discussed above.

The gas phase of the tissue culture is also important and its composition and volume is determined by the type of medium used, the amount of buffering required, and whether

the culture vessel is open or sealed. Common variables include concentration of carbon dioxide and oxygen.

Other conditions important to tissue culture include the choice of culture vessel, amount of headspace, inoculation density, temperature, frequency of media changes, treatment with enzymes, rate and mode of agitation or stirring. In various applications, for instance in studies of cell differentiation, it will often be the case that a specific series of different tissue culture conditions are required to effect a change in phenotype. The different conditions may include additions or withdrawals to/from the media or the change of media at specific time points. Such a set of conditions, examples of which are given below, are commonly developed by trial end error as has been discussed above.

Formation of cell units

An important aspect of the present invention is that groups of cells (cell colonies) can be grown in cell culture under various conditions and that the colony can largely maintain its integrity under various conditions, when disturbed, and when mixed with other colonies. Such groups or colonies are referred to herein as cell units. This may be achieved, by way of illustration, by growing cells as adherent cultures on solid substrates such as glass spheres. In general, live adherent cells are difficult to dissociate from their growth substrate, and so the integrity of the cell colony persists despite any mechanical manipulation of the glass bead, agitation of the culture medium, or transfer into another tissue culture system. Similarly, if at any time multiple beads are placed in the same vessel (i.e. the beads are pooled) then there will be no substantial transfer of cells from one bead to another.

One advantage of growing cells in units or colonies is that if a unit is placed serially in a set of different tissue culture media, then all the cells comprising the colony will have been exposed to the same series of culture conditions, in the same order and for the same period of time. Growing cells in units that are not necessarily themselves adherent to the tissue culture vessel has the advantage that individual colonies can be removed at will and transferred to a different culture vessel. One of the advantages of this method is that tissue culture can be miniaturised: relatively few cells are required to colonise a microcarrier bead (see below) compared to even the smallest tissue culture flask. A further advantage of growing cell units formed on carriers is that cell culture can be scaled up. Growth of stem cells on carriers offers a way of scaling up production to

provide enough material for stem cell therapy. Equally, differentiation of stem cells on carriers offers a way of scaling up production as differentiation proceeds, eventually providing enough material for cell replacement therapies. Scale up of such cell cultures requires at least 50g (dry weight) of microcarrier, preferably, 100g, 500g, 1kg, 10kg or more.

Another important advantage of forming cell units on solid substrates is that the substrate – and therefore the attached cells by reason of association - can be labelled by various means.

Glass spheres of 3mm and 5mm have been widely used as cell adhesion substrates, particularly in glass bead bioreactors (e.g. such as manufactured by Meredos Gmbh) used for the scale-up of cell cultures. These beads are typically used in packed beds rather than batch culture, to avoid mechanical damage to the adherent cells.

In contrast when cells are grown on smaller carriers they can be treated as a suspension culture. Microcarrier cultures are used commercially for antibody and interferon production in fermenters of up to 4000 litres. A variety of microcarriers are available, ranging in shape and size and made of different materials. Microcarrier beads made of polystyrene (Biosilon, Nunc), glass (Bioglass, Solohill Eng), collagen (Biospheres, Solohill Eng), DEAE sephadex (Cytodex-1, Pharmacia), dextran (Dormacell, Pfeifer & Langen), cellulose (DE-53, Whatman), gelatin (Gelibead, Hazelton Lab), and DEAE dextran (Microdex, Dextran Prod.) amongst others are commercially available. These carriers are well characterised in terms of the specific gravity of the beads, the diameter and the surface area available for cell growth. In addition a number of porous (micro) carriers are available with greatly increased surface area for cell growth. A further characteristic of these porous carriers is that they are suitable for growth of both anchorage dependent cells, as well as for suspension cells which are carried by entrapment in the network of open, interconnecting pores. Porous carriers are available in materials such as gelatin (Cultispher G, HyClone), cellulose (Cytocell, Pharmacia), polyethylyne (Cytoline 1 and 2, Pharmacia), silicone rubber (Immobasil, Ashby Scientific), collagen (Microsphere, Cellex Biosciences), and glass (Siran, Schott Glassware). These carriers are variously suited to stirred, fluidised or fixed bed culture systems.

As the physical properties of carriers are well known it is easy to calculate the number of carriers used in an experiment. Some of the carriers described and many besides are available as dried products which can be accurately weighed, and subsequently prepared by swelling in liquid medium. In addition the number of cells used to inoculate a microcarrier culture can be worked out and varied. For instance, a culture of Cytodex 3 (2 g/litre) inoculated at 6 cells per bead will give a culture containing 8 million microcarriers, on which 48 million cells/litre are grown at a density of $5x10^4$ cells/cm^2. Harvesting of cells grown on microcarriers can be achieved by enzymatic detachment of cells, or alternatively by digestion of the carrier itself where applicable: gelatin can be solubilised by trypsin and/or EDTA, collagen beads using collagenase and dextran using dextranase.

In addition to solid or porous microcarriers, cells may be grouped by immurement, i.e. confined within a medium permeable barrier. Membrane culture systems have been developed where a permeable dialysis membrane retains a group of cells, but allows the culture medium and its constituents to exchange freely with the inner and outer compartments. Cell culture in hollow fibre cartridges has also been developed, and a multitude of fibres and even turn-key systems are commercially available (e.g. from Amicon, Cellex Biosciences). Cell encapsulation in semi-solid matrices has also been developed, where cells are immobilised by adsorption, covalent bonding, crosslinking or entrapment in a polymeric matrix. Materials that have been used include gelatin, polylysine, alginate and agarose. A typical protocol, is to mix 5% agarose at 40°C with a suspension of cells in their normal growth medium, to emulsify the mixture using an equal volume of paraffin oil and to cool in an ice bath, producing spheres of 80-200μm diameter. These spheres can be separated from the oil and transferred to medium in a tissue culture vessel.

Cell entrapment is a simple method for the immobilisation of groups of cells, akin to the use of microcarriers or porous substrates. A simple technique is to enmesh cells in cellulose fibres such as DEAE, TLC, QAE, TEAE (all available from Sigma). Other more sophisticated devices are ceramic cartridges which are suitable for suspension cells, as in the Opticel culture system (Cellex Biosciences).

One skilled in the art will envisage, in addition to the above methods of creating cell units, other methods of creating groupings of cells including forming 3D cultures of cells

such as neural spheres or embryoid bodies, or using tissues and indeed whole organisms such as Drosophila or C. Elegans.

Combinatorial serial culture of cells

Split-pool cell culture

Forming cell units (particularly microscopic cell units) is useful for sampling multiple tissue culture conditions as each cell unit constitutes an easily handled unit that can be exposed to a variety of cell culture conditions. For simplicity, in this discussion we will assume that cell groupings are produced by growing cells in microcarrier culture, and the terms cell unit, cell group, colony and bead are used interchangeably. However, the methods described are equally applicable to any cell unit, for instance those described above. A particularly efficient method for sampling a large number of cell culture conditions is referred to as split-pool cell culture (Fig 1). The method operates by taking an initial starter culture (or different starter cultures) of cell units divided into X_1 number of aliquots each containing multiple beads (groups/colonies/carriers) which are grown separately under different culture conditions. Following cell culture for a given time, the cell units can be pooled by combining and mixing the beads from the different aliquots. This pool can be split again into X_2 number of aliquots, each of which is cultured under different conditions for a period of time, and subsequently also pooled. This iterative procedure of splitting, culturing and pooling (or pooling, splitting and culturing; depending on where one enters the cycle) cell units allows sampling of many different cell culture conditions (the combinations of cell culture conditions tested is equal to the product of the number of different conditions (X_1, X_2, ...X_n) sampled at each round). The procedure is efficient because multiple cell units can share a single vessel, where they are cultured under identical conditions, and it can be carried out using only a few culture vessels at any one time (the number of culture vessels in use is equal to the number of split samples). In many respects the principle of this procedure resembles that of split synthesis of large combinatorial libraries (known as combinatorial chemistry), which samples all possible combinations of linkage between chemical building block groups (see for example: Combinatorial Chemistry, Oxford University Press (2000), Hicham Fenniri (Editor)). The split-pool procedure can be repeated over any number of rounds, and any number of conditions can be sampled at each round. So long as the number of cell units (or colonised beads in this example) is greater than or equal to the number of different conditions sampled over all rounds, and assuming that the splitting of cell units occurs totally randomly, it is expected that there will be at least one cell unit that has

been cultured according to each of the various combinations of culture conditions sampled by the experiment. This procedure can be used to sample growth or differentiation conditions for any cell type, or the efficiency of biomolecule production (e.g. production of erythropoeitin or interferon) by any cell type. Because the procedure is iterative, it is ideally suited to testing multistep tissue culture protocols - for instance those described above in connection with stem cell differentiation. The variables which can be sampled using this technique include cell type, cell grouping (e.g. microcarrier culture, cell encapsulation, whole organism), growth substrate (e.g. fibronectin on microcarrier), duration of cell culture round, temperature, different culture media (including different concentrations of constituents), growth factors, conditioned media, co-culture with various cell types (e.g. feeder cells), animal or plant extracts, drugs, other synthetic chemicals, infection with viruses (incl. transgenic viruses), addition of transgenes, addition of antisense or anti-gene molecules (e.g. RNAi, triple helix), sensory inputs (in the case of organisms), and others.

Analysis and/or separation of cell units

Following each round of cell culture, or after a defined number of rounds, the cell units can be assayed to determine whether there are members displaying increased cell proliferation. This can be achieved by a variety of techniques, for instance by visual inspection of the cell units under a microscope, or by quantitating a marker product characteristic of the cell. This may be an endogenous marker such as a particular DNA sequence, or a cell protein which can be detected by a ligand or antibody. Alternatively an exogenous marker, such as green fluorescent protein (GFP), can be introduced into the cell units being assayed to provide a specific readout of (living) cells. Conversely, dead cells can be labelled using a variety of methods, for instance using propidium iodide. Furthermore the labelled cell units can be separated from unlabelled ones by a variety of techniques, both manual and automated, including affinity purification ('panning'), or by fluorescence activated cell sorting (FACS).

Following each round of cell culture, or after a defined number of rounds, the cell units can be assayed to determine whether there are members displaying a particular genotype or phenotype. Genotype determination can be carried out using well known techniques such as the polymerase chain reaction (PCR), fluorescence in situ hybridisation (FISH), DNA sequencing, and others. Phenotype determination can be carried out by a variety of techniques, for instance by visual inspection of the cell units

under a microscope, or by detecting a marker product characteristic of the cell. This may be an endogenous marker such as a particular DNA sequence, or a cell protein which can be detected by a ligand or antibody that recognises a particular phenotypic marker (See Appendix E of *Stem Cells: Scientific Progress and Future Research Directions.* Department of Health and Human Services. June 2001; incorporated herein by reference). The labelled cell units can be separated from unlabelled ones by a variety of techniques, both manual and automated, including affinity purification ('panning'), or by fluorescence activated cell sorting (FACS).Nishikawa et al (1998, Development vol 125, p1747-1757) used cell surface markers recognised by antibodies to follow the differentiation of totipotent murine ES cells. Using FACS they were able to identify and purify cells of the haematopoietic lineage at various stages in their differentiation.

An alternative or complementary technique for enriching cell units of a particular genotype or phenotype is to genetically select the desired groups. This can be achieved for instance by introducing a selectable marker into the cell units, and to assay for viability under selective conditions. Li et al (1998, Curr Biol vol 8, p 971-974) identified neural progenitors by integrating the bifunctional selection marker/reporter βgeo (which provides for β-galactosidase activity and G418 resistance) into the Sox2 locus by homologous recombination in murine ES cells. Since one of the characteristics of neural progenitors is expression of Sox2, and therefore the integrated marker genes, these cells could be selected from non-neuronal lineages by addition of G418 after inducing differentiation using retinoic acid. Cell viability could be determined by inspection under a microscope, or by monitoring β-gal activity. Unlike phenotype-based selection approaches, which can be limited by the availability of an appropriate ligand or antibody, genetic selection can be applied to any differentially expressed gene.

Determination of culture history of a cell unit

When handling large numbers of cell units, their history (for example the chronology and the exact nature of a series of culture conditions that any one group may have been exposed to) can become confused. For instance, the split-pool protocol of cell culture necessarily involves mixing cell units in each round, making it difficult to follow individual units. It is therefore advantageous to label the cell units. This may be achieved by a variety of means, for instance labelling either the cells themselves, or any material to which the cells are attached or otherwise associated with. Any of the chemical and non-chemical methods used to encode synthetic combinatorial libraries can be adapted for

this purpose and some of these are described in Methods in Enzymology Vol 267 (1996), 'Combinatorial Chemistry', John N. Abelson (Editor); and Combinatorial Chemistry, Oxford University Press (2000), Hicham Fenniri (Editor).

The principle of these labeling methods is that unique tags are sequentially associated with the cell units as they are split and/or exposed to each culture condition in a series, such that subsequent detection of the tags provides for an unambiguous record of the chronology and identity of the cell culture conditions to which the cell unit has been exposed. One simple chemical tag that can be introduced to cells or attached to a matrix associated with cells is an oligonucleotide of defined length and/or sequence. Oligonucleotides can be taken up by cells, or attached to the cell surface by a suitable ligand or antibody, or conjugated to a cell-associated matrix by adsorption or a variety of linkages such as covalent linkage or non-covalent linkage, e.g. biotin-streptavidin linkage. The oligonucleotides may comprise any class of nucleic acid (e.g. RNA, DNA, PNA, linear, circular or viral) and may contain specific sequences for amplification (e.g. primer sequences for PCR) or labels for detection (e.g. fluorophores or quenchers, or isotopic tags). The detection of these may be direct, for instance by sequencing the oligos or by hybridising them to complementary sequences (e.g. on an array or chip), or indirect as by monitoring an oligonucleotide-encoded gene product, or the interference of the nucleotide with a cellular activity (e.g. antisense inhibition of a particular gene). In addition to oligonucleotides, any other molecular tag can be used so long as it can be detected, including peptide tags, secondary amines, halocarbons, mixtures of stable isotopes etc. Cell growth substrates such as those described in connection with forming cell units can be derivatised or coated with substances that facilitate tagging and do not interfere with cell growth. In general it will be important to use a tag that can be distinguished from similar molecules present in cells or the culture media, and that can be attached to its target and subsequently detected in the background of such molecules. To facilitate detection, it may be advantageous to selectively elute tags from colonised beads or to strip off the cells from tagged beads using selective conditions. More complicated molecular tagging strategies can also be envisaged, including the strategy of 'binary encoding' where information is recorded by a set of binary codes assigned to a set of molecular tags and their mixtures.

Of particular interest are labelling or encoding strategies which are non-physical and therefore non-invasive. One such strategy uses electronic memory to record the history of a sample. Nicolaou et al (1995, Angew Chem Intl Ed Engl, vol. 34, p. 2289) have

described a microelectronic memory semiconductor where information is recorded via remote radiofrequency transmission. A 'microreactor' incorporating this technology, used to follow the synthesis of a peptide library on beads, comprised: (i) a porous enclosure containing a synthesis substrate and the semiconductor tag; (ii) the solid phase synthesis resin; (iii) a glass encased Single or Multiple Addressable Radiofrequency Tag (SMART) semiconductor unit capable of receiving, storing and emitting radiofrequency signals). A similar device could be adapted to growing and following cell units simply by replacing the solid phase synthesis resin with tissue culture microcarriers or suitable cell units. More variations of this can be envisaged including but not limited to (coated) RF tags on which cells are grown directly, or RF tags implanted into cell units or organisms. In addition, multiple variations of the non-chemical tagging strategy can be devised to determine the identity of a given cell unit in a mixture or of deducing the identity of the different cell units that comprise a mixture. For instance optical or visual methods of tagging have been described where a combination of beads of different colour denote the identity of a sample (for example see 1998, Guiles et al, Angew. Chem. Intl Ed Engl, vol 37, p926), or where a pattern or bar code is etched onto a substrate such as a ceramic bar and recognised using pattern recognition technology (for example see 1997, Xiao et al, Angew. Chem. Intl Ed Engl, vol 36, p780).

The invention is further described in the following examples.

Examples

A split-pool culture experiment was performed in order to assay tissue culture conditions that might give rise to neurons of a dopaminergic phenotype using a starter culture of undifferentiated mouse ES cells.

Undifferentiated ES cells were grown on gelatin-coated tissue culture plates in the presence of 1,400 U ml -1 of leukemia inhibitory factor (LIF; GIBCO/BRL, Grand Island, NY) in ES cell medium consisting of knockout Dulbecco's minimal essential medium (DMEM; GIBCO/BRL) supplemented with 15% FCS, 100 mM MEM nonessential amino acids, 0.55 mM 2-mercaptoethanol, L-glutamine, and antibiotics (all from GIBCO/BRL). To induce embryoid body (EB) formation the cells were dissociated into a single-cell suspension by 0.05% trypsin and 0.04% EDTA in PBS and plated onto nonadherent bacterial culture dishes at a density of 2–2.5 × 10^4 cells cm^{-2} in the medium described above. The EBs were formed for four days and then plated onto adhesive tissue culture

surface in the ES cell medium. After 24 h of culture, selection of nestin-positive cells was accomplished by replacing the ES cell medium by serum-free Insulin/Transferrin/Selenium/Fibronectin (ITSFn) medium and incubating for 10 days.

These nestin-positive cells were used as the starting material for a split-pool culture experiment. Specifically, the cells were dissociated by 0.05% trypsin/0.04% EDTA, and seeded on >10,000 glass biospheres (Whatman, UK) at approximately 1.5–2 x 10^5 cells cm^{-2}. Tissue culture dishes used henceforth were made from non-adherent material. Sterile glass beads were pre-coated using polyornithine (15 mg ml^{-1}) and laminin (1 µg ml^{-1}, both from Becton Dickinson Labware, Bedford, MA). The beads were divided randomly into four sets, each of which was incubated in one of four tissue culture media (denoted A1, A2, A3 or A4) whose composition is given in Table 1 below. These are based on N2 medium modified according to Johe et al. (1996, Genes Dev, vol 10, p 3129-3140) and which ishenceforth referred to simply as N2 medium).

A1	N2 medium supplemented with 1 µg ml^{-1} of laminin.
A2	N2 medium supplemented with 1 µg ml^{-1} of laminin, 10 ng ml^{-1} of bFGF (R&D Systems, Minneapolis, MN).
A3	N2 medium supplemented with 1 µg ml^{-1} of laminin, 10 ng ml^{-1} of bFGF (R&D Systems, Minneapolis, MN), murine N-terminal fragment of SHH (500 ng ml –1, from R&D Systems).
A4	N2 medium supplemented with 1 µg ml^{-1} of laminin, 10 ng ml^{-1} of bFGF (R&D Systems, Minneapolis, MN), murine N-terminal fragment of SHH (500 ng ml -1, R&D Systems) and murine FGF8 isoform b (100 ng ml^{-1}, R&D Systems).

Table 1

The four sets of beads were exposed to the respective tissue culture media for two days and then beads from all four cultures were pooled, washed briefly in N2 medium, and again split into four sets each of which was incubated in one of media A1-A4. After two days this procedure was repeated again, in order to sample cell culture in various combinations of media A1-A4 over a six day period. After this time, beads from all four cultures were pooled, washed briefly in N2 medium, and randomly split into four new sets each of which was incubated in one of four new media (denoted B1, B2, B3 or B4) whose composition is given in Table 2 below.

B1	N2 medium supplemented with 1 µg ml^{-1} of laminin, 10 ng ml^{-1} of bFGF (R&D Systems, Minneapolis, MN), murine N-terminal fragment of SHH (500 ng ml^{-1}, R&D Systems) and

	murine FGF8 isoform b (100 ng ml $^{-1}$, R&D Systems).
B2	N3FL medium: DMEM/F12 (1:1) medium containing insulin (25µg/ml), transferrin (50µg/ml), progesterone (20nM),putrescine (100µM), selenium chloride (30nM), bFGF (5ng/ml) and laminin (1µg/ml),
B3	N2 medium supplemented with 25mM HEPES (pH 7.4), laminin (1 mg ml $^{-1}$), cAMP (1 µM, Sigma, St. Louis, MO) and ascorbic acid (200 µM, Sigma, St. Louis, MO).
B4	N2 medium supplemented with laminin (1 mg ml $^{-1}$), cAMP (1 µM, Sigma, St. Louis, MO) and ascorbic acid (200 µM, Sigma, St. Louis, MO).

Table 2

Each round of culture was for five days, then beads from all four cultures were pooled, washed briefly in N2 medium, and split into four sets each of which was again incubated in one of media B1-B4. This procedure was carried out a total of three times, such that the beads were present in the B media a total of 15 days.

Included in the composition of the different culture media was a unique oligonucleotide label that adhered to the glass microcarriers (or cells) in small quantities and was subsequently amplified and analysed in order to deduce the whereabouts of a microcarrier bead at any time in the split pool culture regimen. The DNA sequence of each label differed so as to distinguish between the different media (i.e. medium A1 vs medium A2) and also exposure to the same medium on two different rounds of split pool culture (i.e. medium A1 used on Day 0 vs on day 4). A summary of the split pool culture regimen in the various media over a total of 21 days is given in Table 3 below. Each entry in the table also shows (in parentheses) the identity of the label included in the tissue culture flask. The full DNA sequence of the label is shown in Table 4.

Day 0	Day 2	Day 4	Day 6	Day 11	Day 16
A1 (L1)	A1 (L5)	A1 (L9)	B1 (L13)	B1 (L17)	B1 (L21)
A2 (L2)	A2 (L6)	A2 (L10)	B2 (L14)	B2 (L18)	B2 (L22)
A3 (L3)	A3 (L7)	A3 (L11)	B3 (L15)	B3 (L19)	B3 (L23)
A4 (L4)	A4 (L8)	A4 (L12)	B4 (L16)	B4 (L20)	B4 (L24)

Table 3

L1	TGCAGGAATTCGCGCTATGCTaACGTGAAGcCACGTCGCCgCCGCCGCCGACCCGGCCGAATTCCTG
L2	TGCAGGAATTCGCGCTATGCTgACGTGAAGtCACGTCGCCaCCGCCGCCGACCCGGCCGAATTCCTG
L3	TGCAGGAATTCGCGCTATGCTcACGTGAAGaCACGTCGCCtCCGCCGCCGACCCGGCCGAATTCCTG
L4	TGCAGGAATTCGCGCTATGCTtACGTGAAGgCACGTCGCCcCCGCCGCCGACCCGGCCGAATTCCTG
L5	TGCAGGAATTCGCGCTATGCTAaCGTGAAGAcACGTCGCCAgCGCCGCCGACCCGGCCGAATTCCTG

L6	TGCAGGAATTCGCGCTATGCTAgCGTGAAGAtACGTCGCCAaCGCCGCCGACCCGGCCGAATTCCTG
L7	TGCAGGAATTCGCGCTATGCTAcCGTGAAGAaACGTCGCCAtCGCCGCCGACCCGGCCGAATTCCTG
L8	TGCAGGAATTCGCGCTATGCTAtCGTGAAGAgACGTCGCCAcCGCCGCCGACCCGGCCGAATTCCTG
L9	TGCAGGAATTCGCGCTATGCTAAaGTGAAGACcCGTCGCCACgCCGCCGACCCGGCCGAATTCCTG
L10	TGCAGGAATTCGCGCTATGCTAAgGTGAAGACtCGTCGCCACaCCGCCGACCCGGCCGAATTCCTG
L11	TGCAGGAATTCGCGCTATGCTAAcGTGAAGACaCGTCGCCACtCCGCCGACCCGGCCGAATTCCTG
L12	TGCAGGAATTCGCGCTATGCTAAtGTGAAGACgCGTCGCCACcCCGCCGACCCGGCCGAATTCCTG
L13	TGCAGGAATTCGCGCTATGCTAACaTGAAGACAcGTCGCCACCgCCGCCGACCCGGCCGAATTCCTG
L14	TGCAGGAATTCGCGCTATGCTAACgTGAAGACAtGTCGCCACCaCCGCCGACCCGGCCGAATTCCTG
L15	TGCAGGAATTCGCGCTATGCTAACcTGAAGACAaGTCGCCACCtCCGCCGACCCGGCCGAATTCCTG
L16	TGCAGGAATTCGCGCTATGCTAACtTGAAGACAgGTCGCCACCcCCGCCGACCCGGCCGAATTCCTG
L17	TGCAGGAATTCGCGCTATGCTAACGaGAAGACACcTCGCCACCGgCGCCGACCCGGCCGAATTCCTG
L18	TGCAGGAATTCGCGCTATGCTAACGgGAAGACACtTCGCCACCGaCGCCGACCCGGCCGAATTCCTG
L19	TGCAGGAATTCGCGCTATGCTAACGcGAAGACACaTCGCCACCGtCGCCGACCCGGCCGAATTCCTG
L20	TGCAGGAATTCGCGCTATGCTAACGtGAAGACACgTCGCCACCGcCGCCGACCCGGCCGAATTCCTG
L21	TGCAGGAATTCGCGCTATGCTAACGTaAAGACACGcCGGCACCGCgGCCGACCCGGCCGAATTCCTG
L22	TGCAGGAATTCGCGCTATGCTAACGTgAAGACACGtCGCCACCGCaGCCGACCCGGCCGAATTCCTG
L23	TGCAGGAATTCGCGCTATGCTAACGTcAAGACACGaCGCCACCGCtGCCGACCCGGCCGAATTCCTG
L24	TGCAGGAATTCGCGCTATGCTAACGTAAGACACGgCGCCACCGCcGCCGACCCGGCCGAATTCCTG

Table 4

By split-pool culturing the stem cells three times in the different A media followed by three times in the different B media it was possible to sample 4096 different tissue culture protocols - this being the total number of different combinations of the above buffers to which different beads were exposed.

Following the final round of split pool culture the beads were pooled, washed briefly in N2 medium, and analysed by FACS using standard protocols. Briefly, cells were fixed in 4% paraformaldehyde/0.15% picric acid in PBS. In order to detect dopaminergic neurons, cells were stained using an anti-tyrosine hydroxylase monoclonal (Sigma) followed by a fluorescently labelled secondary antibody (Jackson Immunoresearch Laboratories, West Grove, PA) both according to the manufacturer's instructions.

The five beads with the highest fluorescence intensity were sorted into separate wells of a PCR plate and the oligonucleotide labels were amplified by thirty cycles of PCR using Taq polymerase (Stratagene, La Jolla, CA) and the primers shown in Table 5 below.

PRF1	TGCAGGAATTCGCGCTATGC
PRR2	CAGGAATTCGGCCGGGTCGG

Table5

The PCR products were purified by phenol/chloroform extraction followed by ethanol precipitation, digested using the restriction enzyme EcoRI (New England Biolabs, Beverley, MA) and cloned into similarly prepared pBluescript II KS+ vector (Stratagene, La Jolla, CA). The recombinant vector was electroporated into competent E. coli DH5α and plated on medium containing ampicillin antibiotic. Two hundred colonies were picked for each bead analysed and plasmid DNA prepared and sequenced in the region of the polylinker.

The sequencing analysis revealed that the majority of beads carrying cells with a dopaminerdic phenotype were labeled by oligos (L4, L8, L12, L16, L20 and L24). Correlating these labels to their respective cell culture conditions suggested that these beads had been partitioned in medium A1 over a total of six days (on Day 0, Day 2 and Day 4) followed by medium B4 for a further fifteen days (on Day 6, Day 11 and Day 16). It was deduced that this culture protocol was one appropriate for the production of dopaminergic neurons by processing mouse ES cells as described above.

Once these conditions were established, undifferentiated ES cells were again cultured according to the above protocol (i.e expansion, EB formation, nestin selection etc.) but without undergoing the process of split pool culture. Cells were grown on adhesive tissue culture plates instead of beads, oligonucleotide labels were not added to the different culture media, and only the successful culturing conditions were assayed. In this way larger numbers of dopaminergic cells could be produced and tested for patterns of gene expression. Total RNA was removed from cells obtained from four stages of the culture protocol: (1) pluripotent ES cell population; (2) embryoid bodies; (3) dissociated and plated cells undergoing selection for nestin; and (4) nestin positive cells cultured in buffer A4. cDNA was prepared using reverse transcriptase and priming with random hexamers and the amount of actin transcript normalised between the various samples. Neural cDNAs were prepared using the primers shown in Table 6:

GENE	FORWARD PRIMER	REVERSE PRIMER	+ve STAGE
Otx2	CCATGACCTATA CTCAGGCTTCAGG	GAAGCTCCATATCC-CTGGGTGGAAAG	1, (2, 3), 4
Pax2	CCAAAGTGGTGGACAAGATTGCC	GGGATAGGAAGGACGCTCAAAGAC	3, 4
Pax5	CAGATGTAGTCCGC-CAAAGGATAG	ATGCCACTGATGGAGTATGAGGAGCC	3, 4
En1	TCAAGACTGACTCACAGCAACCCC	CTTTGTCCTGAACCGTGGTGGTAG	4
Wnt1	ACCTGTTGACGGATTCCAAG	TCATGAGGAAGCGTAGGTCC	3, 4
Num1	TGAAGAGAGCGGAGAAGGAGATC	TCTGGAGTTAAGAAATCGGAGCTG	3, 4
nestin	GGAGTGTCGCTTAGAGGTGC	TCCAGAAAGCCAAGAGAAGC	(1), 3, 4

Table 6

Table 6 also shows the stages in which transcripts of the various genes were detected by RT-PCR (parentheses indicate detection of traces). From these results it was deduced that expression of En1, Pa 2, Pax5, Wnt1 and Nurr1 would be a suitable marker for cells destined to a dopaminergic fate. Conversely, transfection of pluripotent stem cells with such genes, for example Nurr1, could result in commitment to a dopaminergic phenotype in vitro (Wagner et al, 1999, Nature Biotechnology, vol. 17, p 653-659).

CLAIMS

1. A method for determining the effect of a plurality of culture conditions on a cell, comprising the steps of:

a) providing a first set of groups of cell units each comprising one or more cells, and exposing said groups to desired culture conditions;

(b) pooling two or more of said groups to form at least one second pool;

(c) subdividing the second pool to create a further set of groups of cell units;

(d) exposing said further groups to desired culture conditions;

(e) optionally, repeating steps (b) – (d) iteratively as required; and

(f) assessing the effect on a given cell unit of the culture conditions to which it has been exposed.

2. method for determining the effect of a plurality of culture conditions on a cell, comprising the steps of:

(a) providing a first set of groups of cell units each comprising one or more cells, and exposing said groups to desired culture conditions;

(b) subdividing one or more of said groups to create a further set of groups of cell units;

(c) exposing said further groups to further desired culture conditions;

(d) optionally, repeating steps (b) – (c) iteratively as required; and

(e) assessing the effect on a given cell unit of the culture conditions to which it has been exposed.

3. A method according to claim 1 or claim 2 wherein each cell unit is labelled and the label reflects the sequence of culture conditions to which the cell unit has been exposed.

4. A method according to any preceding claim, wherein the label is spatially encoded.

5. A method according to any one of claims 1 to 3, wherein the label is selected from the group consisting of an oligonucleotide, a peptide, a secondary amine, a halocarbon, a mixture of stable isotopes, an optical tag and a radiofrequency encoding tag.

6. A method according to any preceding claim, wherein the cells are cultured in cell units, each cell unit comprising one or more cells.

7. A method according to claim 6, wherein the cell units are single cells.

8. A method according to claim 6, wherein each cell unit comprises one or more cells adherent to or bounded by a solid substrate.

9. A method according to claim 8, wherein the solid substrate is a microcarrier or bead.

10 A method according to claim 8, wherein the solid substrate is a well or medium-permeable barrier.

11. A method according to any preceding claim, wherein the culture conditions are media to which the cell is exposed.

12. A method according to claim 11, wherein the media contain one or more specific agents which influence cell proliferation and/or differentiation.

13. A method according to any preceding claim, wherein the cell culture conditions comprise culturing at one or more specific temperatures.

14. A method according to any preceding claim, wherein the cell culture conditions comprise culturing on one or more specific substrates.

15. A method for identifying a gene which influences cell proliferation and/or differentiation, comprising the steps of:

a) determining the effect of one or more culture conditions on a cell unit, in accordance with any one of the preceding claims;

b) analysing gene expression in said cell units when exposed to said culture conditions; and

c) identifying genes which are differentially expressed under desired culture conditions.

16. A method according to claim 15, wherein the desired culture conditions influence cell differentiation and/or proliferation.

17. A method for producing a nucleic acid which encodes a gene product which influences cell proliferation and/or differentiation, comprising identifying a gene in accordance with claim 15 or claim 16, and producing at least the coding region of said gene by nucleic acid synthesis or biological replication.

18. A method for inducing differentiation and/or proliferation in a cell, comprising the steps of:

a) identifying one or more genes which are differentially expressed in association with cell differentiation and/or proliferation in accordance with claim 15 or claim 16; and

b) modulating the expression of said one or more genes in the cell.

19. A method according to claim 18, wherein modulation of gene expression in the cell comprises transfection of said one or more genes into the cell.

20. A method according to claim 18, wherein modulation of gene expression comprises the exogenous administration of a gene product.

21. A method for identifying the differentiation state of a cell, comprising the steps of:

a) identifying one or more genes which are differentially expressed in association with cell differentiation in accordance with claim 15 or claim 16; and

b) detecting the modulation of expression of said one or more genes in a cell, thereby determining the differentiation state of said cell.

22. A method according to claim 21, wherein said one or more genes encode an extracellular marker.

23. A method according to claim 22, wherein said extracellular marker may be detected by an immunoassay.

24. A method for producing a differentiated cell, comprising the steps of:

a) determining the effect of one or more culture conditions on a cell unit, in accordance with any one of claims 1 to 14;

b) exposing a pluripotent or totipotent cell to culture conditions which induce the desired differentiation; and

c) isolating the desired differentiated cell.

25. A method for identifying an agent which is capable of inducing cell differentiation, comprising the steps of:

a) determining the effect of one or more agents on a cell unit, in accordance with any one of claims 1 to 14; and

b) identifying those agent(s) which induce the desired differentiation in the cell units.

26. A method for preparing an agent which is capable of inducing cell differentiation, comprising the steps of:

a) determining the effect of one or more agents on a cell unit, in accordance with any one of claims 1 to 14;

b) identifying those agent(s) which induce the desired differentiation in the cell units; and

c) synthesising or isolating the agent(s).

27. A method for culturing stem cells or cells that have been derived from stem cells in vitro, comprising the steps of:

a) combining one or more cultures of cells grown under different conditions; and

b) culturing the cells.

28. A method for culturing stem cells or cells that have been derived from stem cells in vitro comprising the steps of:

a) incubating a stem cell culture; and

b) splitting said culture into two or more groups of stem cells, and culturing said group of stem cells under two or more different sets of culture conditions.

29. A method according to claim 27 or claim 28, wherein the cells are cultured in cell units, each cell unit comprising one or more cells.

30. A method according to claim 27 or claim 28, wherein the cell units are single cells.

31. A method according to claim 27 or claim 28, wherein each cell unit comprises one or more cells adherent to or bounded by a solid substrate.

32. A method according to claim 31, wherein the solid substrate is a microcarrier or bead.

33. A method according to claim 31 wherein the solid substrate is a well or medium-permeable barrier.

34. A method for culturing stem cells, comprising growing said stem cells adhered to a microcarrier or bead.

35. A method according to claim 34, wherein said stem cells are subjected to at least one change of culture conditions.

36. A method according to claim 35, wherein said change of culture conditions comprises a change of medium.

37. A method according to any one of claims 34 to 36, wherein the process is scaled up such that at least 50g (dry weight) of microcarrier is employed.

38. A method for obtaining differentiated cells from stem cells in vitro, comprising the steps of:

(a) Growing stem cells adherent to microcarriers in a culture medium;
(b) Transferring the microcarriers from one culture medium to another;
(c) Optionally repeating step (b) as required; and
(d) Obtaining the differentiated cells attached to the microcarrier.

39. A method according to the preceding claim, wherein the process is scaled up such that at least 50g (dry weight) of microcarrier is employed.

40. A method according to claim 38 or 39, wherein the differentiated cells are isolated by enzymatic detachment from the microcarrier.

41. A method according to claim 38 or 39, wherein the differentiated cells are isolated by digestion of the microcarrier.

Abstract

A method for determining the effect of a plurality of culture conditions on a cell, comprising the steps of: a) providing a first set of groups of cell units each comprising one or more cells, and exposing said groups to desired culture conditions; (b) pooling two or more of said groups to form at least one second pool; (c) subdividing the second pool to create a further set of groups of cell units; (d) exposing said further groups to desired culture conditions; (e) optionally, repeating steps (b) – (d) iteratively as required; and (f) assessing the effect on a given cell unit of the culture conditions to which it has been exposed.

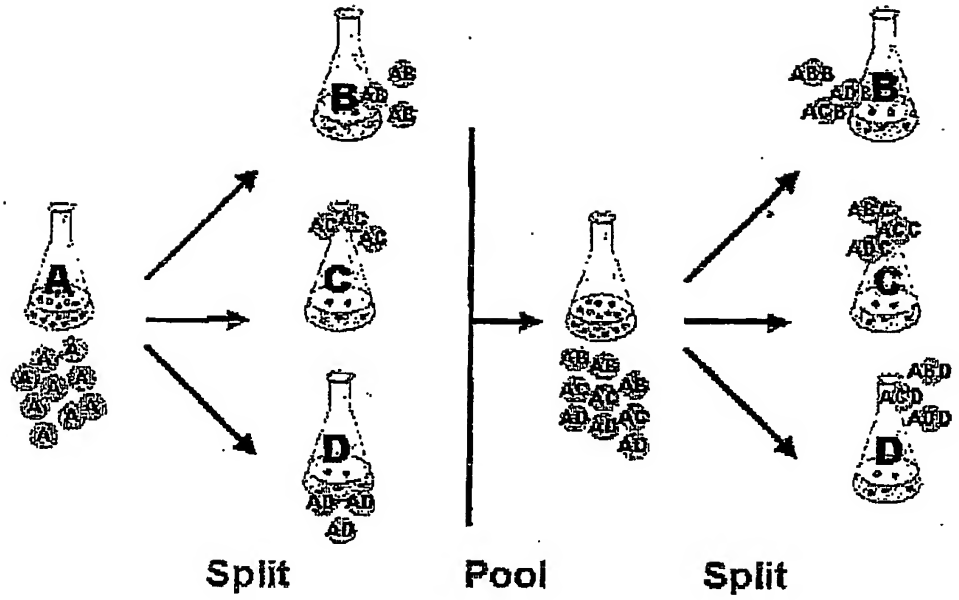

Figure 1

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